Ureaplasma parvum undergoes selection in utero resulting in genetically diverse isolates colonising the chorioamnion of fetal sheep

Running Title: In utero selection of *U. parvum* variants

Summary Sentence: The chorioamnion selects for different ureaplasma sub-types within a non-clonal population during chronic intrauterine infection; ureaplasmas isolated from chorioamnion tissue contained highly polymorphic 23S rRNA gene sequences.

Keywords: ureaplasma, amniotic fluid, chorioamnion, ribosomal RNA, minimum inhibitory concentration, ovine model.

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Abstract

Ureaplasmas are the microorganisms most frequently isolated from the amniotic fluid of pregnant women and can cause chronic intrauterine infections. These tiny bacteria are thought to undergo rapid evolution and exhibit a hypermutatable phenotype; however, little is known about how ureaplasmas respond to selective pressures in utero. Using an ovine model of chronic intra-amniotic infection, we investigated if exposure of ureaplasmas to sub-inhibitory concentrations of erythromycin could induce phenotypic or genetic indicators of macrolide resistance. At 55 days gestation, 12 pregnant ewes received an intra-amniotic injection of a non-clonal, clinical *U. parvum* strain, followed by: (i) erythromycin treatment (IM, 30 mg/kg/day, n=6); or (ii) saline (IM, n=6) at 100 days gestation. Fetuses were then delivered surgically at 125 days gestation. Despite injecting the same inoculum into all ewes, significant differences between amniotic fluid and chorioamnion ureaplasmas were detected following chronic intra-amniotic infection. Numerous polymorphisms were observed in domain V of the 23S rRNA gene of ureaplasmas isolated from the chorioamnion (but not the amniotic fluid), resulting in a mosaic-like sequence. Chorioamnion isolates also harboured the macrolide resistance genes *erm*(B) and *msr*(D) and were associated with variable roxithromycin minimum inhibitory concentrations. Remarkably, this variability occurred independently of exposure of ureaplasmas to erythromycin, suggesting that low-level erythromycin exposure does not induce ureaplasmal macrolide resistance in utero. Rather, the significant differences observed between amniotic fluid and chorioamnion ureaplasmas suggest that different anatomical sites may select for ureaplasma sub-types within non-clonal, clinical strains. This may have implications for the treatment of intrauterine ureaplasma infections.

Introduction

The human ureaplasmas (*Ureaplasma parvum* and *Ureaplasma urealyticum*) are among the smallest self-replicating bacteria, typically ranging in size from 100 nm to 1 μm [1]. They can be isolated from the mucosal surfaces of the vagina or cervix in 40 – 80% of sexually active females [2] and are the most frequently isolated microorganisms from infected amniotic fluids and placentas [3-6]. Although amniotic fluid contains a number of bacteriostatic/bacteriocidal components [7], ureaplasmas have been detected in the amniotic fluid of pregnant women as early as the 16th week of pregnancy (in the presence of intact fetal membranes) and have been reported to persit for as long as two months [8]. Furthermore, in a sheep model of intra-amniotic infection, we demonstrated that ureaplasmas can colonise the amniotic fluid for up to 85 days [9]. Although often clinically silent, intra-amniotic ureaplasma infections stimulate a pro-inflammatory host response [9, 10] and are associated with histological chorioamnionitis, funisitis and preterm birth. Ureaplasmas may also colonise the fetus in utero, or be vertically transmitted to the infant at birth, and are associated with neonatal diseases including bronchopulmonary dysplasia, pneumonia, sepsis and meningitis [11, 12].

Eradication of intra-amniotic ureaplasma infections by antimicrobial treatment is thought to improve pregnancy outcomes and reduce neonatal morbidity and mortality [13, 14]. Erythromycin (a 14-membered lactone ring macrolide) is routinely administered to pregnant women for the treatment of intra-amniotic infections and preterm, prelabour rupture of membranes. However, this treatment may be ineffective [15, 16] as there is minimal placental transfer of erythromycin from the maternal circulation to the amniotic fluid. In humans, the placental transfer of erythromycin is as low as 3% [17], suggesting that microorganisms within the amniotic fluid might only be exposed to low levels of antimicrobials. In an ovine model of intra-amniotic ureaplasma infection, we reported that standard-dose maternal

erythromycin treatment achieved low concentrations (<10 – 76 ng/mL) in the amniotic fluid and did not eradicate infection [18].

Exposure of bacteria to non-lethal concentrations of antimicrobials may promote antimicrobial resistance, which can occur by target site modification, drug efflux pumps, or drug inactivation mediated by short peptides [19]. Mechanisms of macrolide resistance that have been identified in ureaplasmas include target site modification via mutations in the 23S rRNA gene and ribosomal protein L4 and L22 genes [20-22]. The lactone ring of macrolide antimicrobials interacts hydrophobically with the crevice formed by bases 2057, 2058 and 2059 (*Escherichia coli* numbering) of the 23S rRNA gene; therefore a mutation in any of these nucleotides may inhibit macrolide binding. Similarly, point mutations in ribosomal protein L4 and L22 genes may also allosterically affect macrolide binding [19]. In addition, erythromycin-ribosome methylase (*erm*) genes, which post-translationally methylate nucleotide 2058 of domain V of the 23S rRNA gene to inhibit macrolide binding via steric hindrance, have been reported in ureaplasmas and may be associated with phenotypic resistance [23]. Macrolide resistance may also occur by the activity of drug efflux pumps such as the macrolide streptogramin resistance (*msr*) genes, which export antimicrobials out of the bacterial cell. Previously, Lu *et al.* [23] detected *msr*(A), *msr*(B) and *msr*(D) subtypes in *Ureaplasma* spp. and suggested that they may be associated with ureaplasmal resistance to macrolides and/or lincosamides.

Members of the *Mycoplasmataceae* family with very small genomes, such as the *Ureaplasma* spp., exhibit a hypermutatable phenotype and may undergo rapid evolution [24, 25]. Indeed, ureaplasmas undergo significant genetic variability associated with: (i) size/phase variation of surface-exposed antigens [26-30]; (ii) the presence of hypervariable plasticity zones functioning as putative pathogenicity islands [31] and (iii) horizontal gene transfer and proposed recombination events resulting in genetic mosaics [32, 33]. Furthermore, point

mutations in ureaplasmal 23S rRNA, ribosomal protein L4 and L22 genes can be induced in vitro by passaging isolates in broth medium containing sub-inhibitory concentrations of macrolide antimicrobials [22]. However, to the best of our knowledge, the effects of low-level antimicrobial exposure on ureaplasmas have not been investigated in vivo.

Using an ovine model of chronic intra-amniotic infection, we tested if *U. parvum* colonising the amniotic fluid and chorioamnion of pregnant sheep underwent genetic variation following exposure to selective antimicrobial pressure. Specifically, we investigated if exposure of ureaplasmas to sub-inhibitory concentrations of erythromycin in utero could induce genetic markers of macrolide resistance. By sequencing regions of the ureaplasmal 23S rRNA gene and performing PCRs to detect macrolide resistance genes, we observed significant genetic variability within chorioamnion ureaplasmas following chronic, intra-amniotic infection.

Materials and Methods

Ethics statement

All experimental procedures involving animals were performed in accordance with the "Australian code of practice for the care and use of animals for scientific purposes" (National Health and Medical Research Council of Australia) and were approved by the Animal Ethics Committee of The University of Western Australia.

Animal model and specimen collection

The inoculum used for intra-amniotic injection was a low-passage, erythromycin-susceptible, *U. parvum* serovar 3 isolate (442S) that was originally isolated from the semen of an infertile man attending the Wesley IVF Service (Brisbane, Queensland). Isolate 442S was selected for this study as it was a non-clonal, clinical strain containing a mixture of ureaplasma subtypes that were adherent and non-adherent to the surface of spermatozoa. A non-clonal strain, as opposed to a clonal ureaplasma strain that had been cloned and filtered in vitro, was chosen to closely model ureaplasmas isolated from natural infections. Ureaplasmas for injection were prepared as previously described and diluted to 2×10^4 CFU in PBS prior to intra-amniotic injection [34].

The samples analysed in this study were collected from a previously described experiment [18]. Briefly, at 55 days gestation (term = 150 days gestation), 12 date-mated Merino ewes bearing single fetuses received a 2 mL intra-amniotic injection of *U. parvum* 442S [34]. At 100 days gestation, ewes were randomly assigned to receive erythromycin treatment (*Up*/E group; n = 6) or saline (*Up* group; n = 6). Ewes that received erythromycin treatment were injected intra-muscularly with 500 mg of erythromycin (Abbot Australasia, Kurnell, New South Wales) three times daily for four days (100 – 104 days gestation), resulting in a total

dose of 30 mg/kg/day. Additional antibiotics were not added to the supplementary feed given to pregnant ewes, nor did animals receive antimicrobial treatment as part of on-going veterinary care. Preterm fetuses were surgically delivered at 125 days gestation [18]. Samples of amniotic fluid and chorioamnion were aseptically collected and stored at -80 °C for subsequent analysis.

Ureaplasma culture

Ureaplasmas were cultured from amniotic fluid and chorioamnion samples collected from each animal. Thawed chorioamnion tissue (0.1 g) was homogenised in 1.5 mL of 10B medium [35] using a mini beadbeater 8-cell disrupter (Daintree Scientific, St Helens, Tasmania). Homogenised chorioamnion and amniotic fluid samples were then cultured in 10B medium [36] and positive cultures were stored at -80 °C for further analysis.

23S rRNA, ribosomal protein L4 and ribosomal protein L22 PCR and sequencing

To detect polymorphisms within genes associated with macrolide resistance, PCRs targeting domain II and domain V of the 23 rRNA gene, ribosomal protein L4 gene and ribosomal protein L22 gene were performed on the ureaplasma isolates from the amniotic fluid and chorioamnion. DNA was extracted from first passage cultures in 10B broth using previously described methods [37]. The PCR assays were performed in 50 µL reaction mixtures containing: 100 µM of dNTP mix (Roche Diagnostics, Castle Hill, New South Wales), 1 x PCR buffer (Invitrogen, Mt Waverley, Victoria), 1.5 mM of $MgCl_2$ (Invitrogen), 0.5 µM of each primer (Sigma Aldrich, Castle Hill, New South Wales; primer sequences are shown in Table 1), 2.5 U of Platinum *Taq* Polymerase (Invitrogen) and PCR-grade H_2O. PCR cycling involved initial denaturation at 94 °C for 15 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, primer annealing at 56 °C for 1 minute, extension at 72 °C for 2 minutes, plus a final extension at 72 °C for 15 minutes.

PCR products from *U. parvum* 442S and representative amniotic fluid (n = 5) and chorioamnion (n = 4) strains were purified using the High Pure PCR Product Purification Kit (Roche). Sequencing reactions were performed by the Australian Genome Research Facility (St Lucia, Queensland). Sequence data were trimmed to obtain sequences of a uniform length and then aligned using Geneious Pro version 5.6.5. Partial 23S rRNA sequences from representative amniotic fluid and chorioamnion ureaplasma isolates were deposited in GenBank (National Center for Biotechnology Information, 2013) under the accession numbers JF521483, JF521484, JF521485 and JF521486.

erm(B) and *msr* gene PCR

PCRs were performed to detect selected genes associated with macrolide resistance (*erm*(B), *msr*(A), *msr*(B), *msr*(C) and *msr*(D)) [23, 38-40]. These assays were performed on isolates selected for 23S rRNA and ribosomal protein L4 and L22 gene sequencing. PCRs were performed in 50 µL volumes as described above, using primers shown in Table 1. PCR reactions were performed using cycling conditions described by Lu et al. [23]; however, initial denaturation and final elongation steps were modified to 94 °C for 15 minutes and 72 °C for 15 minutes respectively.

Minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of erythromycin (Sigma Aldrich), roxithromycin (Sigma Aldrich) and azithromycin (Pfizer, West Ryde, New South Wales) were determined for ureaplasmas isolated from amniotic fluid and chorioamnion samples at 125 days gestation. Isolates to be tested were thawed and diluted to a standardised concentration of 1 x 10^4 CFU/mL in 10B broth. MICs were determined using standard broth microdilution assays [41, 42] and interpreted according to defined CLSI breakpoints [42].

Each antibiotic was tested in triplicate and MIC results are expressed as the mean value of the three experiments.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism version 5.0. A two-tailed Fisher's exact test was used to compare the proportion of amniotic fluid and chorioamnion ureaplasma isolates harbouring macrolide resistance genes. MIC data were compared between *Up* and *Up*/E groups using independent, two-tailed t tests.

Results

Intra-amniotic ureaplasmas caused chronic intrauterine infection

Injection of *U. parvum* into the amniotic fluid of pregnant ewes at 55 days gestation resulted in chronic intrauterine infection. At 125 days gestation, ureaplasmas were isolated from 6//6 amniotic fluid samples (100%) and 4/6 chorioamnion samples (67%) collected from the *Up* group. Maternal erythromycin treatment (*Up*/E group) did not eliminate ureaplasma colonisation within the amniotic fluid (6/6 (100%) culture positive) and chorioamnion (4/6 (67%) culture positive) at 125 days gestation. Quantitative bacterial cultures from these amniotic fluid and chorioamnion samples were reported in a previous publication [18]. First passage cultures were stored at -80 °C and used for DNA extraction and MIC testing.

23S rRNA gene sequence variability in ureaplasmas isolated from the chorioamnion

The maternal administration of erythromycin resulted in sub-inhibitory antimicrobial levels within the amniotic fluid in pregnant sheep [18]. To determine if ureaplasmas exposed to sub-inhibitory concentrations of erythromycin in utero demonstrated genetic markers of antimicrobial resistance, PCR was performed to amplify domain II and domain V of the 23S rRNA gene, and the genes encoding ribosomal protein L4 and ribosomal protein L22 (data not shown). Amplicons from amniotic fluid and chorioamnion ureaplasma isolates, and *U. parvum* isolate 442S were then sequenced. Within each of the sequenced regions, *U. parvum* isolate 442S shared 100% sequence identity with the *U. parvum* serovar 3 reference strain (ATCC 700970; Genbank accession number AF222894). No sequence polymorphisms were detected in any of the ureaplasmas, which were isolated from amniotic fluid samples, across any of the targeted regions of the 23S rRNA gene and the L4 and L22 ribosomal protein genes. Hence, all amniotic fluid isolates shared 100% sequence identity with the *U. parvum* serovar 3 reference strain and isolate 442S (the inoculum).

Conversely, significant genetic variability was detected within all ureaplasma isolates from the chorioamnion, when compared to amniotic fluid ureaplasma isolates, *U. parvum* ATCC 700970 and *U. parvum* 442S (Table 2). These polymorphisms occurred independently of exposure to erythromycin as they were detected in chorioamnion isolates from both the *Up* and *Up*/E groups. The regions of genetic variability were localised within domain V of the 23S rRNA gene, specifically within the regions amplified by PCR primers MH23S-11/MP23S-22 and MH23S-9/MP23S-23. Within the region amplified by the primer pair MH23S-11/MP23S-22, 72 identical polymorphisms, 5 insertions and 5 deletions (out of 230 sequenced nucleotides) were detected in each chorioamnion ureaplasma isolate. Similarly, within the region of domain V of the 23S rRNA gene amplified by PCR primer MH23S-9/MP23S-23, 36 identical polymorphisms (out of 200 nucleotides) were detected. Within these polymorphic regions, a higher G+C content were also observed (Table 2). Gene alignments demonstrating the significant sequence variability between chorioamnion and amniotic fluid ureaplasma strains are shown in Figure 1 (MH23S-11/MP23S-22 amplicon) and Figure 2 (MH23S-9/MP23S-23 amplicon).

Despite the large number of polymorphisms detected in chorioamnion ureaplasma strains, specific nucleotides that were previously associated with macrolide resistance in ureaplasmas and other bacteria (nucleotides G2056, G2057 and A2058 of domain V of the 23S rRNA gene, *E. coli* numbering [22]; and C2243, *U. urealyticum* numbering [21]) remained conserved. No polymorphisms were detected in domain II of the 23S rRNA gene or ribosomal proteins L4 and L22.

Macrolide resistance genes were detected in ureaplasmas isolated from chorioamnion tissue

The macrolide resistance gene *erm*(B) was not detected in any ureaplasmas isolated from the amniotic fluid, but was present in *U. parvum* 442S and 100% of ureaplasmas isolated from the chorioamnion ($p = 0.008$; Figure 3A). Of the four tested *msr* gene subtypes, *msr*(D) was the only gene detected in the ureaplasma isolates (Figure 3B). *msr*(D) was detected in *U. parvum* 442S, 2/5 amniotic fluid ureaplasma isolates (40%) and 100% of chorioamnion ureaplasma isolates ($p < 0.05$). The presence of macrolide resistance genes occurred independently of exposure to erythromycin, as *erm*(B) and *msr*(D) genes were detected in ureaplasmas from both the *Up* and *Up*/E groups.

23S rRNA genetic variability and *erm*(B) and *msr*(D) genes were not associated with phenotypic resistance to macrolide antimicrobials

The inoculum strain used for this animal study, *U. parvum* strain 442S, was susceptible to erythromycin, azithromycin and roxithromycin, with MIC values of 0.13 mg/L, 0.5 mg/L and 0.5 mg/L respectively. Ureaplasmas isolated from the amniotic fluid after 125 days gestation also demonstrated susceptibility to erythromycin, azithromycin and roxithromycin (Table 3). The MICs of these three antimicrobials were not different when tested against amniotic fluid ureaplasmas isolated from the *Up* group and the *Up*/E group ($p > 0.05$).

Although ureaplasmas isolated from the chorioiamnion demonstrated: (i) significant genetic variability within domain V of the 23S rRNA gene, and (ii) the presence of *erm*(B) and *msr*(D) resistance genes, these isolates were not phenotypically resistant to the tested macrolide antimicrobials (Table 3). The MICs of erythromycin and azithromycin against ureaplasmas isolated from the chorioamnion were low, ranging from 0.06 – 0.25 mg/L, whereas the MICs of roxithromycin were variable against chorioamnion ureaplasmas, ranging from 0.13 – 5.33 mg/L. However, according to the defined CLSI breakpoints, none of the chorioamnion ureaplasma isolates were resistant to roxithromycin. The MICs of

erythromycin, azithromycin and roxithromycin were not statistically different between ureaplasmas isolated from the chorioamnion from the *Up* group and the *Up*/E group ($p > 0.05$).

Discussion

We investigated whether low-level erythromycin exposure could induce genetic markers of macrolide resistance in ureaplasmas isolated from the amniotic fluid and chorioamnion of pregnant sheep at 125 days gestation. Sub-inhibitory erythromycin concentrations were achieved in the amniotic fluid via maternal intra-muscular erythromycin treatment at 100 – 104 days gestation [18]. Previous studies have suggested that ureaplasmas are highly variable and undergo rapid evolution [24, 25]; therefore we hypothesised that the application of selective antimicrobial pressure for a short period of time (four days) may be sufficient to generate genetic variants. We assessed this by performing PCR and sequencing of domain II and domain V of the ureaplasmal 23S rRNA gene and ribosomal protein L4 and L22 genes. Mutations in these genes were previously associated with macrolide resistance in ureaplasmas [20-22]. We detected significant genetic variability, including nucleotide substitutions, insertions and deletions, within domain V of the 23S rRNA gene; however, this variability did not occur in response to erythromycin treatment. Rather, genetic variability within the 23S rRNA gene was detected exclusively in ureaplasmas isolated from the chorioamnion following chronic intrauterine infection and was present in ureaplasmas isolated from both the treatment and the control group animals.

Genetic diversity was detected within two regions of domain V of the 23S rRNA gene, amplified by two separate primer pairs. *U. parvum* possesses two ribosomal operons, both of which were amplified simultaneously by the primers used in this study. Sequence chromatograms did not reveal double peaks at any loci, indicating that both copies of the 23S rRNA gene were identical. Marques et al. [43] previously reported intraspecific sequence variation of the 16S rRNA gene of the bovine pathogen *U. diversum*. In their study of 34 field isolates, four hypervariable regions within the 16S rRNA gene were identified and within these regions 44 polymorphisms were detected. Furthermore, an isolate with only 88%

sequence similarity to the *U. diversum* 16S rRNA reference sequence was identified. In the present study, across the two variable regions of domain V of the 23S rRNA gene, ureaplasmas isolated from the chorioamnion shared only 78% sequence similarity with the *U. parvum* 442S inoculum strain.

Due to: (i) the presence of identical polymorphisms in chorioamnion ureaplasma strains and (ii) the increased G+C content of these variable regions, we propose that these regions of genetic variability may represent fragments transferred by horizontal gene transfer (HGT). Previous studies have demonstrated that ureaplasmas undergo extensive HGT resulting in genetic mosaics, which arise from proposed recombination events [32, 33]. *U. parvum* isolate 442S, the non-clonal inoculum strain, was characterised in our laboratory and found to contain a mixed population of ureaplasmas with different phenotypic properties [44]. We suggest that this strain may also contain a small population of 23S rRNA genetic variants, which were generated via previous HGT events. However, sequencing of domain V of the 23S rRNA gene of isolate 442S did not detect the variant sequence nor were double peaks observed at any loci. Xiao et al. demonstrated that mixed populations of ureaplasmas could not be detected by Sanger sequencing once the DNA concentration ratio reached 9:1 [32]. Thus it is likely that the variant sequence is present only in a small, undetectable population of ureaplasmas within isolate 442S, which were subsequently selected for in the chorioamnion during chronic intra-amniotic infection.

Previous studies have shown that the host immune response and host genetic background impact the outcomes of ureaplasma infection [9, 45]. Here, we have demonstrated that ureaplasma subtypes within a mixed population may be selected for at different anatomical sites within a host, which may also affect outcomes. We are unable to speculate as to which components of the chorioamnion may have resulted in the selection of the variant ureaplasma subtype; however, there are significant differences in the microenviromnent of the amniotic

fluid compared to that of the chorioamnion, which may result in different selective pressures between these anatomical sites. The amniotic fluid is a proteinaceous biological fluid, which undergoes dynamic change througout pregnancy. Early in gestation, the protein content of amniotic fluid resembles that of maternal serum (albeit at lower concentrations); however, fetal urine is a major component of amniotic fluid in the second half of pregnancy [46]. A proteomic analysis of human amniotic fluid demonstrated that amniotic fluid contained proteins that function in immune defence, cell communication/transport, metabolism, enzyme activity, signal transduction, development/cell differentiation, cell proliferation, cell organisation and others of unknown function [47]. Although comprehensive proteomic studies of the chorioamnion are lacking, the chorioamnion contains mutliple cellular layers, connective tissue and an extracellular matrix, which is composed largely of collagen [48]. The source of innate immune cells within the amniotic fluid and chorioamnion have also been shown to differ – neutrophils within the chorioamnion are maternally-derived, whereas amniotic fluid neutrophils are fetal in origin [49, 50]. Interestingly, Namba et al. [50] demonstrated that sulfoglycolipid, the receptor for *Ureaplasma* spp., is localised within the amnion suggesting that the chorioamnion may have selected for adherent ureaplasma sub-types in our study, although this remains to be confirmed.

It is remarkable that identical variant populations were expanded within the chorioamnion of all experimentally-infected animals. However, similar findings were previously reported for other microorganisms. In an in vitro model of *Escherichia coli* evolution, two populations derived from a common ancestor were propagated for 20, 000 generations in minimal medium [51]. Identical changes in gene expression were detected in the two independently propagated *E. coli* populations relative to the ancestral strain, demonstrating parallel evolution and/or selection. In vivo selection of variant populations of *Burkholderia pseudomallei* has also been demonstrated. Complete genome sequencing of human *B.*

pseudomallei stains isolated: (i) during primary infection and (ii) following relapse of the primary infection [52] or from persistent asymptomatic carriage [53] showed that strong selection of genetic variants occurred during chronic infection. Tissue-specific selection has also been reported for *Pseudomonas aeruginosa* and *Staphylococcus aureus* colonising the lungs of cystic fibrosis patients [54, 55]. An *in silico* study of the coding sequences of *U. urealyticum* and the related microorganisms *Mycoplasma genitalium* and *M. pneumoniae* suggested that genetic selection at the codon level for *U. urealyticum* and *M. genitalium* is most likely to be driven by environmental stimuli rather than phylogenetic relationships [56]. Here, we provide evidence that different anatomical sites may select for ureaplasma variants and thus alter the socio-microbiological structure of the bacterial population.

Although chorioamnion ureaplasma isolates demonstrated significant genetic variability within domain V of the 23S rRNA gene, these isolates were not phenotypically resistant to erythromycin, azithromycin or roxithromycin. Heterogeneity was observed in the MICs of roxithromycin when tested against ureaplasmas isolated from the chorioamnion, with MIC values ranging from 0.13 – 5.33 mg/L. Despite this, according to recently defined CLSI breakpoints, MICs ≤4 mg/L indicate susceptibility, whereas MICs ≥16 mg/mL indicate resistance to macrolide antimicrobials. Therefore, none of the tested isolates were resistant to the tested macrolides. We also tested the ability of chrorioamnion ureaplasma isolates to form biofilms in vitro; however, biofilm formation was not associated with increased resistance to the erythromycin, azithromyin or roxithromycin, compared to planktonic chorioamnion ureaplasma isolates (data not shown). Nucleotides that were previously associated with macrolide resistance in ureaplasmas [21, 22] remained conserved in all tested strains, potentially explaining why ureaplasmas isolated from the chorioamnion were susceptible to macrolides. It is surprising that such significant changes in the sequence of domain V of the 23S rRNA gene were not associated with increased resistance to macrolides, due to potential

changes in the secondary structure of the rRNA. Furthermore, we did not observe any differences in growth rates between ureaplasma isolates containing the wild type rRNA sequence and those with the variant rRNA sequence (data not shown), suggesting that genetic variability within 23S rRNA did not affect ureaplasmal fitness. This is in agreement with Asai et al. [57] who reported that following inactivation of all seven *E. coli* chromosomal rRNA operons and insertion of foreign rRNA operons derived from *Salmonella typhimurium* or *Proteus vulgaris*, no effects on microbial fitness were observed. Collectively, these data demonstrate that bacteria may swap fragments or entire rRNA operons via HGT without significant impact on survival.

We also performed PCR for the amplification of macrolide resistance genes including *erm*(B), *msr*(A), *msr*(B), *msr*(C) and *msr*(D). Previously, Lu et al. [23] characterised macrolide and lincosamide resistant ureaplasma strains and reported that ureaplasmas harbouring the *erm*(B) gene were associated with erythromycin MICs ranging from 8 - ≥128 mg/L. The presence of *erm*(B) was also significantly associated with the *int-Tn* gene, which is a genetic marker of a transposon, and it was suggested that *erm*(B) may be part of a ureaplasmal transposon. Here, we detected *erm*(B) in isolate 442S and all chorioamnion ureaplasma isolates, but not in any ureaplasmas isolated from the amniotic fluid. This provides further evidence that a variant ureaplasma population within isolate 442S was selected for within the chorioamnion. In contrast to Lu et al., in our study *erm*(B) was not associated with phenotypic resistance to macrolides. We also detected *msr*(D) in isolate 442S, 40% of amniotic fluid ureaplasmas and 100% of chorioamnion ureaplasmas. Similar to previous findings in which *msr*(D) was associated with very wide MIC ranges and did not always confer a resistant phenotype [23], our data demonstrated that the presence of *msr*(D) was not associated with macrolide resistance. Interestingly, the *msr*(D) PCR produced additional faint bands in chorioamnion ureaplasma isolates that were not present in amniotic

fluid ureaplasmas or isolate 442S. We were unable to identify these bands; however, we speculate that the genetic changes selected for in chorioamnion ureaplasmas may contain multiple *msr*(D) primer binding sites that are not present in the amniotic fluid isolates or the inoculum strain. Further research is required to characterise the role of the *erm* and *msr* genes in ureaplasmas.

A limitation of our study is that ureaplasmas were injected directly into the amniotic fluid of pregnant sheep, therefore the model does not represent the progression of an ascending invasive infection from the lower genital tract, which is predicted to be a common mechanism of intra-amniotic infection. Ascending infections occur when vaginal microorganisms cross the cervix, ascend into the choriodecidual space, and then cross the chorioamnion and enter the amniotic fluid [58]. However, upper genital tract infections may also arise due to iatrogenic needle contamination at the time of amniocentesis or chronic villus sampling or haematogenous spread through the placenta [58]. Furthermore, ureaplasmas may also access the female upper genital tract by attachment to the surface of spermatozoa [44], or they may be present in the endometrium of healthy, non-pregnant females [11]. Thus ureaplasmas may colonise the amniotic fluid and chorioamnion by mechanisms other than ascending invasive infections.

In summary, in vivo exposure of a non-clonal, clinical ureaplasma strain to sub-inhibitory levels of erythromycin did not induce genetic markers of macrolide resistance. However, we demonstrated significant differences between ureaplasmas isolated from the amniotic fluid and chorioamnion following chronic intrauterine infection in a sheep model, with respect to 23S rRNA gene sequence and the presence of macrolide resistance genes. These findings suggest that different anatomical sites may select for variant populations within a non-clonal ureaplasma strain, and may have several implications. Firstly, the significant genetic differences between amniotic fluid ureaplasmas and chorioamnion ureaplasmas support

previous studies suggesting that ureaplasmas undergo HGT, which may result in the lateral transfer of antibiotic resistance genes, virulence genes or genes required for survival in niche environments. Secondly, the selection of variant ureaplasma subtypes at different anatomical sites suggests that intrauterine ureaplasma infections are highly complex and that amniotic fluid cultures alone may not isolate ureaplasmas that are representative of the entire population present within the fetal compartment. Clinically, this becomes important when considering antimicrobial susceptibility testing and treatment, and may explain why ureaplasma infections can be difficult to eradicate. Finally, these findings also have implications for our understanding of ureaplasmal pathogenesis, as ureaplasma subtypes localised within the chorioamnion and amniotic fluid may be associated with differences in virulence, and differences in the severity of in utero inflammation, which have been observed previously in an animal model [29, 30]. Although not examined in this study, in a previous experiment using the same inoculum strain (isolate 442S), we demonstrated that ureaplasmas isolated from the chorioamnion of pregnant sheep produced a significantly different multiple banded antigen profile compared to ureaplasmas isolated from the amniotic fluid and fetal lung [30]. This provides further evidence that the amniotic fluid and chorioamnion may select for different ureaplasma subtypes within a mixed population. The data presented in this study demonstrate that, similar to related *Mycoplasmas* spp., ureaplasma populations are dynamic and are influenced by the local micro-environment.

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Figure Legends

Figure 1: Significant genetic variability observed in the MH23S-11/MP23S-22 amplicon within domain V of the 23S rRNA gene of ureaplasmas isolated from the chorioamnion at 125 days gestation

Sequence polymorphisms occurred within regions of domain V of the 23S rRNA gene amplified by primers MH23S-11/MP23S-22. Sequence alignment compares representative amniotic fluid (AF) ureaplasma isolates (n=5) and chorioamnion (CAM) ureaplasma isolates (n=4) from the *Up* and *Up*/E groups to the *Ureaplasma parvum* serovar 3 reference strain (*U. parvum* ATCC 700970, Genbank accession number AF222894) and the inoculum strain (*U. parvum* 442S). Numbering shown is *U. parvum* ATCC 700970 23S rRNA numbering. Sequences from strains AF 227 and CAM 227 have been deposited in Genbank (accession numbers JF521483 and JF521484 respectively).

Figure 2: Significant genetic variability observed in the MH23S-9/MP23S-23 amplicon within domain V of the 23S rRNA gene of ureaplasmas isolated from the chorioamnion at 125 days gestation

Sequence polymorphisms occurred within regions of domain V of the 23S rRNA gene amplified by primers MH23S-9/MP23S-23. Sequence alignment compares representative amniotic fluid (AF) ureaplasma isolates (n=5) and chorioamnion (CAM) ureaplasma isolates (n=4) from the *Up* and *Up*/E groups to the *Ureaplasma parvum* serovar 3 reference strain (*U. parvum* ATCC 700970, Genbank accession number AF222894) and the inoculum strain (*U. parvum* 442S). Numbering shown is *U. parvum* ATCC 700970 23S rRNA numbering. Sequences from strains AF 227 and CAM 227 have been deposited in Genbank (accession numbers JF521485 and JF521486 respectively).

Figure 3: Macrolide resistance genes detected in chorioamnion ureaplasma isolates

PCR detection of *erm*(B) **(A)** and *msr*(D) **(B)** genes in ureaplasmas isolated from the amniotic fluid and chorioamnion of pregnant sheep after 125 days gestation. M = Molecular weight marker VII (Roche, Castle Hill, New South Wales); AF = amniotic fluid ureaplasma isolates; CAM = chorioamnion ureaplasma isolates; 442S = *U. parvum* strain 442S; bp = base pairs; C = no template negative control.

PRIMER TARGET AND NAME	SEQUENCE (5' – 3')	REFERENCE
23S Domain V		
23SF	GTGAAATCCTGGTGAGGGTGA	Dongya *et al.* 2008 [21]
23SR	TTCCTACGGGCATGACAGATAG	
MH23S-11	TAACTATAACGGTCCTAAGG	Pereyre *et al*. 2007 [22]
MP23S-22	GGCGACCGCCCCAGTCAAAC	
MH23S-9	GCTCAACGGATAAAAGCTAC	Pereyre *et al*. 2007 [22]
MP23S-23	ACACTTAGATGCTTTCAGCG	
23S Domain II		
Up23S-30	TGCCTTTTGAAGTATGAGCC	Pereyre *et al.* 2007 [22]
Up23S-31	TGGCGCCATCATAGATTCAG	
Ribosomal protein L4 gene		Pereyre *et al.* 2007 [22]
UpL4-U	TCTATTGATGGTAACTTCGG	
UpL4-R	GTTGAAGGTGTTTCTAAATCGC	
Ribosomal protein L22 gene		Pereyre *et al.* 2007 [22]
UpL22-U	TTCGCACCGTAAAGCTTCTC	
UpL22-R	GTTCTGGATCAACGTTTTCG	
***erm*(B)**	GAAAAGGTACTCAACCAAATA	Graham *et al.* 2009 [39]
	AGTAACGGTACTTAAATTGTTTAC	
***msr*(A)**	GGCACAATAAGAGTGTTTAA	Lina *et al*. 1999 [40]
	AAGTTATATCATGAATAGATTGTCCTGTT	
***msr*(B)**	TATGATATCCATAATAATTATCCAATC	Lina *et al*. 1999 [40]
	AAGTTATATCATGAATAGATTGTCCTGTT	
***msr*(C)**	AAGGAATCCTTCTCTCTCCG	Lu *et al.* 2010 [23]
	GTAAACAAAATCGTTCCCG	
***msr*(D)**	TTGGACGAAGTAACTCTG	Daly *et al.* 2004 [38]
	GCTTGGCTCTTACGTTC	

Table 1: PCR primers used for the amplification and sequencing of the 23S rRNA gene and ribosomal protein genes; and the detection of macrolide resistance genes.

	Region amplified by MH23S-11/MP23S-22 PCR primers		Region amplified by MH23S-9/MP23S-23 PCR primers	
	Amniotic fluid ureaplasmas[§]	**Chorioamnion ureaplasmas**	**Amniotic fluid ureaplasmas**[§]	**Chorioamnion ureaplasmas**
Number of polymorphisms compared to 442S[‡]	0/230 nucleotides	72/230 nucleotides	0/200 nucleotides	36/200 nucleotides
Number of insertions compared to 442S[‡]	0/230 nucleotides	5/230 nucleotides	0/200 nucleotides	0/200 nucleotides
Number of deletions compared to 442S[‡]	0/230 nucleotides	5/230 nucleotides	0/200 nucleotides	0/200 nucleotides
Percentage sequence similarity to 442S[‡]	100%	64.3%	100%	82.0%
G+C content	44%	52%	52%	56%
A+T content	56%	48%	48%	44%

Table 2: Significant genetic variability was detected within regions of domain V of the 23S rRNA gene of ureaplasmas isolated from the chorioamnion of pregnant sheep after 125 days gestation. [‡]The number of polymorphisms, insertions, deletions and percentage sequence similarity were calculated relative to the inoculum strain for this study, *U. parvum* serovar 3 isolate 442S. [§]No genetic variability was observed in amniotic fluid ureaplasma isolates when compared to isolate 442S or *U. parvum* serovar 3 ATCC 700970.

		AMNIOTIC FLUID ISOLATES			CHORIOAMNION ISOLATES		
ANIMAL NUMBER	**TREATMENT GROUP**	***MIC (mg/L)***			***MIC (mg/L)***		
		ERY	**AZM**	**ROX**	**ERY**	**AZM**	**ROX**
229	*Up*	0.33	0.50	0.17	0.25	0.25	5.33
230	*Up*	0.08	0.50	0.06	-	-	-
231	*Up*	0.17	0.13	0.13	0.13	0.06	0.67
232	*Up*	0.08	0.33	0.50	0.06	0.06	0.25
233	*Up*	0.35	0.25	0.50	-	-	-
234	*Up*	0.33	0.33	0.50	0.25	0.25	0.50
MIC_{50}		0.17	0.33	0.17	0.13	0.06	0.50
MIC_{90}		0.33	0.50	0.50	0.25	0.25	0.67
222	*Up*/E	0.17	0.33	0.50	-	-	-
223	*Up*/E	0.13	0.50	0.50	-	-	-
225	*Up*/E	0.13	0.13	0.13	0.06	0.13	0.13
226	*Up*/E	0.25	0.29	0.34	0.06	0.17	4.00
227	*Up*/E	0.63	0.72	0.83	0.13	0.10	2.67
228	*Up*/E	0.25	1.00	0.42	0.08	0.06	0.13
MIC_{50}		0.17	0.33	0.42	0.06	0.10	0.13
MIC_{90}		0.25	0.72	0.50	0.08	0.13	2.67

Table 3: MIC values of erythromycin (ERY), azithromycin (AZM) and roxithromycin (ROX) against amniotic fluid and chorioamnion ureaplasma isolates. *Up* = ureaplasma group; *Up*/E = ureaplasma + erythromycin group; dash (-) indicates samples which were ureaplasma culture negative after 125 days gestation.

Figure 1

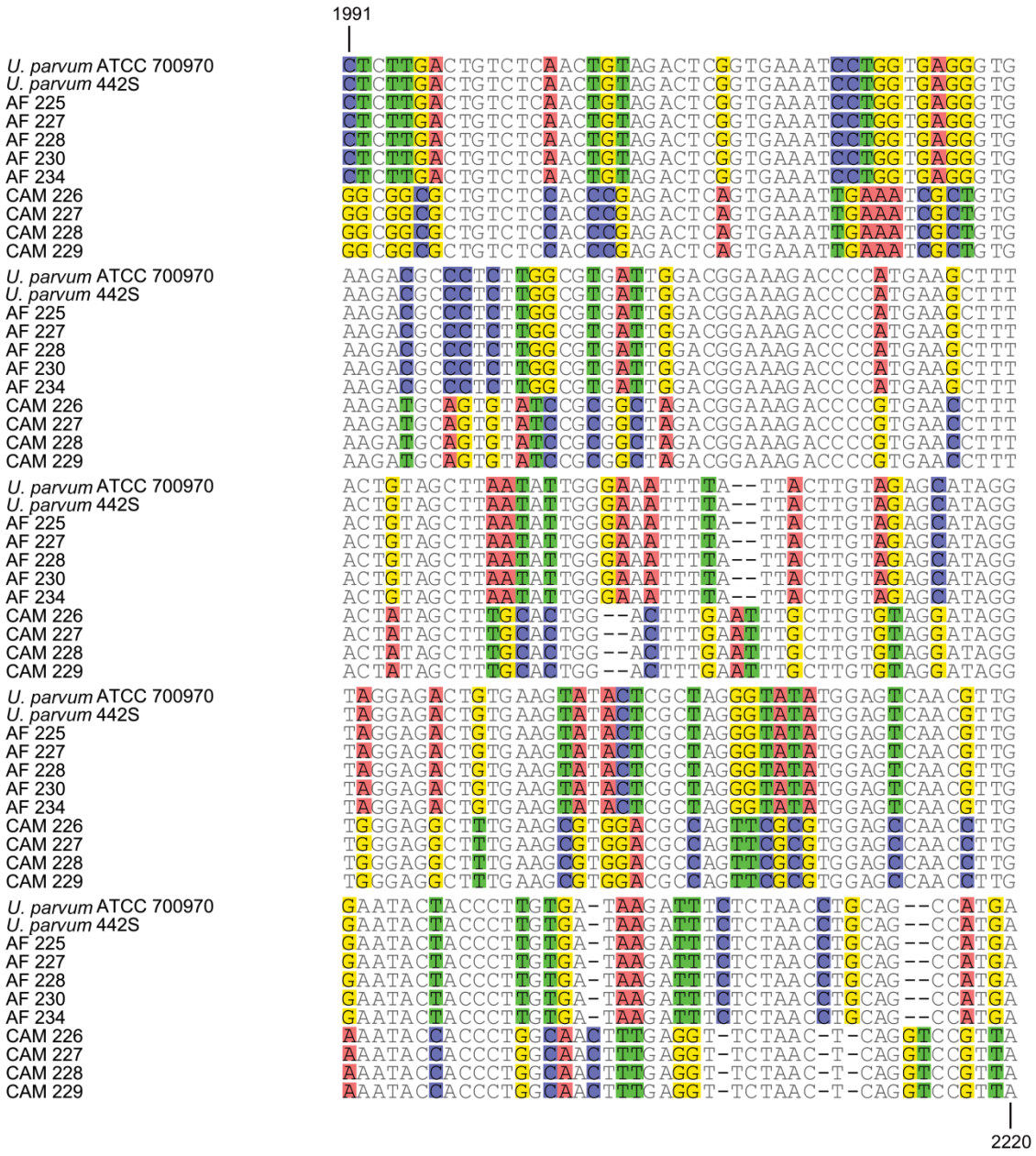

1991
U. parvum ATCC 700970
U. parvum 442S
AF 225
AF 227
AF 228
AF 230
AF 234
CAM 226
CAM 227
CAM 228
CAM 229
2220

Figure 2

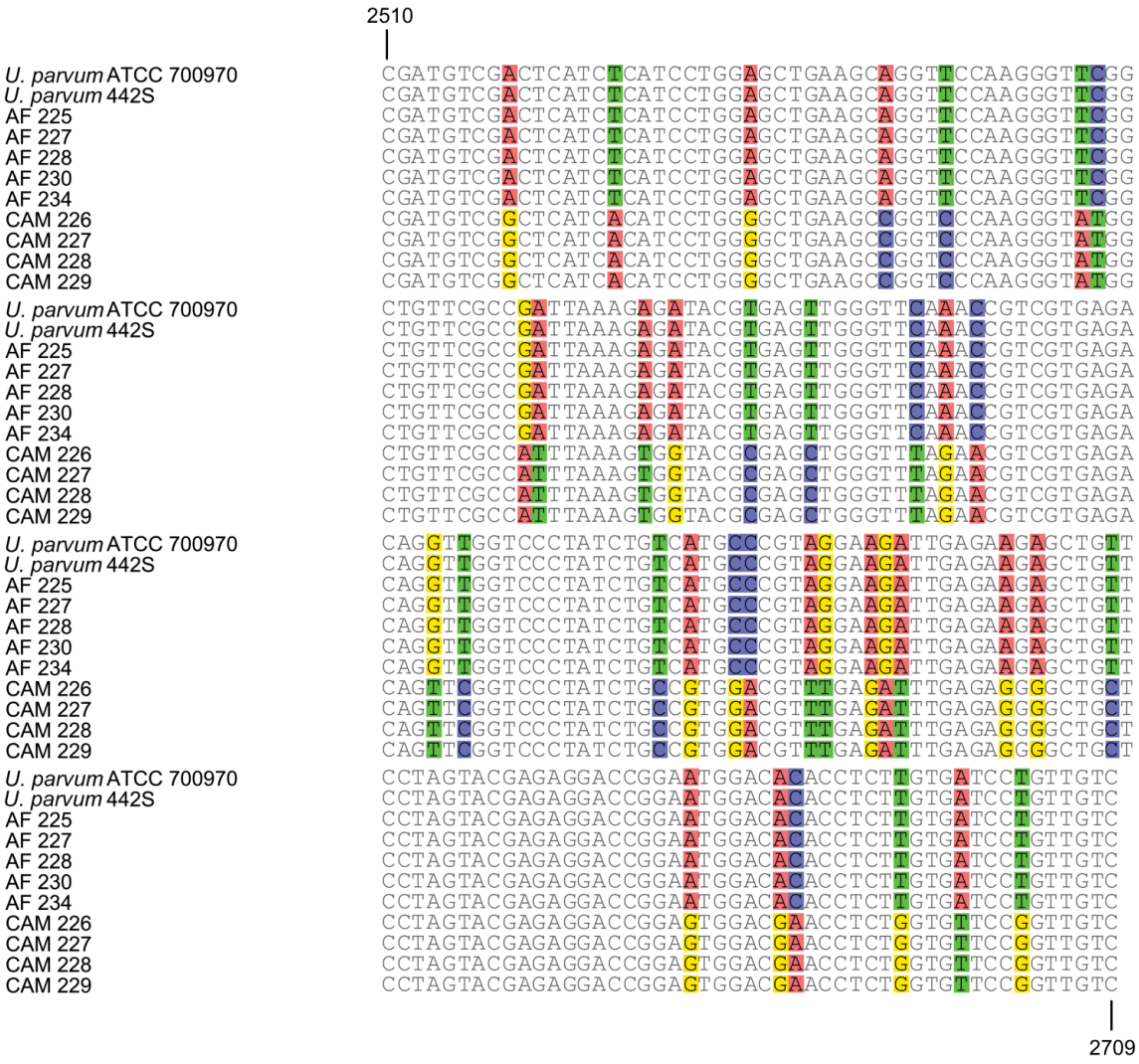
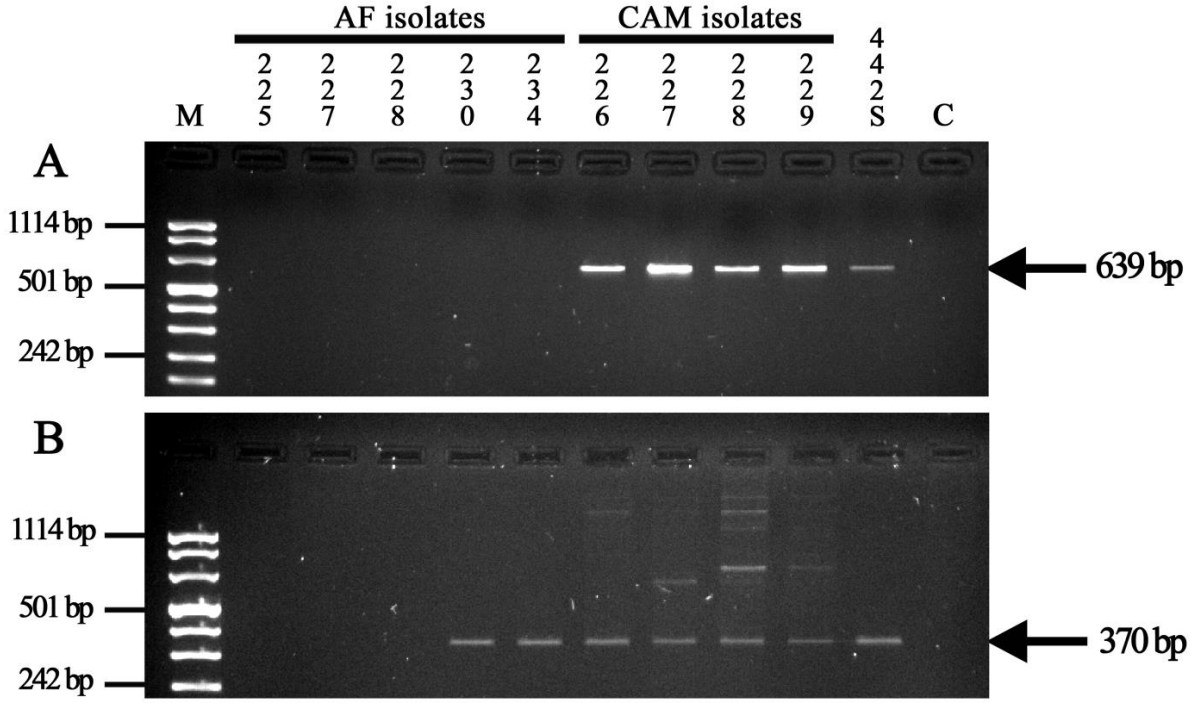

2510
U. parvum ATCC 700970
U. parvum 442S
AF 225
AF 227
AF 228
AF 230
AF 234
CAM 226
CAM 227
CAM 228
CAM 229
2709

Figure 3


AF isolates
CAM isolates
M
225
227
228
230
234
226
227
228
229
442S
C
A
1114 bp
501 bp
242 bp
639 bp
B
1114 bp
501 bp
242 bp
370 bp